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Flow Cytometric Analysis of T and Tn Epitopes on Chronic Lymphocytic Leukemia Cells

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Immunophenotyping of peripheral blood lymphocytes from six patients with B-cell chronic lymphocytic leukemia (B-CLL) and five normal volunteers was done and their T and Tn epitopes analyzed using specific monoclonal antibodies and flow cytometry. Lymphocytes from all patients showed strong Tn expression as compared to normal control lymphocytes. By contrast, T antigen was not expressed. The Tn expression may be a useful diagnostic and prognostic marker for B-CLL. © 1996 Wiley-Liss, Inc.

Key words: B-cell chronic lymphocytic leukemia, T, Tn, flow cytometry, monoclonal antibody

INTRODUCTION

The immediate precursor of the human blood group MN antigens, the Thomsen-Friedenreich (T) antigen, was discovered by Thomsen, Friedenreich, and Huebener in the late 1920s [1]. The structure is normally not immunoreactive because it is covered with sialic acid or O-glycosidic carbohydrate complexes. T-antigen may become unmasked on red blood cells due to sialidase-producing microbes; thus, it is transitory in vivo. This may result in panagglutination caused by the anti-T antibodies present in all normal human sera.

In contrast to T, the Tn antigen is persistent [2]. In Tn^+ individuals, the Tn antigen can be detected on red blood cells, platelets, granulocytes, white cells, and stem cells. Tn antigen is caused by a hemizygous pleiotropic somatic mutation or gene suppression in adults at the pluripotent stem cell level, which may result in the Tn syndrome [3]. Springer et al. [4] discovered that T and Tn were greatly expressed in breast, lung and pancreatic and other carcinomas (CAs), and that specific cellular and quantitative humoral responses to the antigens are found in CA patients. This increased expression is thought to be a result of incomplete synthesis [4], resulting in accumulation of precursor structures. These EPs are considered pancarcinoma (panCA) markers, as they are found in about 90% of all carcinomas [4].

Our knowledge concerning the expression of T and Tn on leukemic cells is limited [5–11]. It was shown that Tn expression may precede acute leukemia and thus may be a predictor of malignancy [2]. Bird et al. [7] showed that Tn polyagglutination occurred in a patient with acute myelocytic leukemia [7] and in a patient with myelofibrosis and autoimmune hemolytic anemia [8]. Berdinskikh et al. [9] suggested that leukocytes from leukemia patients have T antigens. Using polyclonal antisera, Springer et al. [10,11] showed the presence of Tn and T on various T-lymphoblastic and erythroleukemic cell lines. Tn expression on red blood cells has also been shown to occur in B-cell lymphoma [12] and myelodysplasia

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; CA, carcinoma; EP, epitope; HP, *Helix pomatia,* Roman snail lectin; HSM, hepatosplenomegaly; LAP lymphadenopathy; MAb, monoclonal antibody; PNA, peanut agglutinin, *Arachis hypogaea.*

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TABLE I. Clinical Profile of Patients With CLL

Patient/ sample Date	Age (yr)	Sex	Clinical duration of disease	Leukocyte count (/mm³)	Percent lymphocytes (%)	Absolute lymph count	Hgb (g/dl)	Platelet count (/mm³)	Symptoms	Spleen	Rai stage	Previous treatment	Date previous treatment
CLL #1 3/11/91	55	M	3.5 yr (exp. 3/18/91)	55,000	99	54,450	10.1	53,000	Fatigue, LAP, HSM	+	3	Chlorambucil, prednisone, CTX	Nov. 1990
CLL #2 5/2/91	58	M	13 yr	41,700	92	38,364	15.0	142,000	0	0	0	None	None
CLL #3 4/9/91	65	M	2 mo	25,000	44	11,000	15.9	141,000	0	0	0	None	None
CLL #3 5/8/91	65	M	3 mo	ND	ND	ND	16.3	143,000	0	0	0	None	None
CLL #3 7/30/91	65	M	6 mo	ND	ND	ND	ND	ND	0	0	0	None	None
CLL #4 5/2/91	76	M	7 yr	12,500	77	9,625	15.3	220,000	0	0	0	None	None
CLL #5 4/18/91	78	M	7 yr	20,200	ND	ND	13.1	232,000	0	0	1	None	None
CLL #6 7/30/91	72	M	4 yr	16,300	74	12,062	13.7	220,000	0	0	0	None	None

LAP, lymphadenopathy, HSM, hepatosplenomegaly; ND, not determined; CTX, cytoxan (cyclophosphamide).

[13]. Asialo-GM_1 has been shown to have T activity [14], in 1980, asialo-GM_1 was detected as a surface marker in acute lymphocytic leukemia [15]. Tn was recently found to be expressed on a T-lymphoid cell line Jurkat, which is derived from an acute lymphocytic leukemia [16], as well as in myelodysplasia, lymphoma, and leukemia [17].

The purpose of the present investigation was to analyze the expression of T and Tn antigens in B-CLL cells by flow cytometry using monoclonal antibodies (MAbs) and to compare the pattern of expression with that found in normal lymphocytes.

MATERIALS AND METHODS

Patient Population

The study included six patients with B-CLL: five of whom were untreated and one who received treatment. The treated patient had had no chemotherapy during the 4 weeks prior to lymphocyte collection. The diagnosis was based on characteristic clinical, peripheral blood, and bone marrow findings. Profiles of these subjects are given in Table I. Clinical and laboratory data were retrieved directly from the patient's charts at the Veteran's Affairs Medical Center, North Chicago, Illinois.

Clinical Material

Peripheral whole blood samples from B-CLL patients and normal adult volunteers were aseptically collected by venipuncture in sterile 10-ml Vacutainer tubes (Becton-Dickinson, Raritan, NJ) containing sodium heparin. All samples were stored at room temperature until lymphocyte isolation within 24 hr.

Preparation of Lymphocytes

Lymphocytes were separated from peripheral blood by Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density-gradient centrifugation [18]. Whole blood was diluted with RPMI 1640 medium (Gibco, Grand Island, NY); 10 ml was then layered over 4 ml of Ficoll-Paque. After centrifugation at 400*g* for 30 min at 25°C, the interfacial layer containing enriched mononuclear cells was removed, washed three times in RPMI 1640, and resuspended at a concentration of $1–2 \times 10^7$ cells/ml.

Samples not immediately analyzed were frozen in liquid nitrogen until analysis; this has been shown not to affect immunological markers for lymphocytic leukemias adversely [19]. Cryopreservation was performed after resuspension of the cells in RPMI 1640 plus 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). An additional 5% FBS was then added, the bring the final concentration of FBS to 15%. After mixing, 10% dimethyl sulfoxide (DMSO) was added to the cell suspension. The final concentration was $2–3 \times 10^7$ cells/ml. Aliquots of 1.8 ml were placed in vials that were frozen in a polystyrene box at −70°C for 24 hr and then transferred to liquid nitrogen. This method allowed an initial cooling rate of 1–2°C/min. Before use, the cells were quickly thawed by immersion in water at 37°C with gentle agitation. They were then washed twice, resuspended in phosphate-buffered saline (PBS), and then used for flow cytometric

TABLE II. Commercial Antibodies Used in This Study

CD designation	Antigen molecular weight	Monoclonal antibody	Normal cellular reactivity/specificity	Normal range (%) in blood
CD3	22–28K	T3[a]	T-cell receptor complex	68–82
CD5	67K	T1[a]	T-cells, B-cell subset	65–79
CD10	100K	J5[a]	CALLA, Pre-B, Granulocytes	>90 Common ALL
CD11b	170/95K	Mo1[a]	Mac I adhesion molecule Monocytes, granulocytes CR3	8–22
CD14	80K	My4[a]	Monocytes	>85 Monocytes
CD19	95K	B4[a]	Pan-B, functions in B-cell activation	5–15
CD20	35K	B1[a]	B-cells, dendritic reticulum cells	5–15
CD25	55–60K	IL-2R[a]	Anti-interleukin-2 receptor, activated B and T cells	15–40
CD45	180–220K	KC56[a]	Leukocyte common antigen	>95
CD71	180K	Antitransferrin receptor[b]	Lymphoblasts, common tumor cell lines, monocytes Nucleated erythroid cells	100 Lymphoblasts
No cluster designation	62K	IgD[c]	Mature resting B cells	50–70 B cells
No cluster designation	70K	IgM[c]	Mature resting B cells	50–70 B cells
No cluster designation	50K	IgG[c]	Serum IgG	0–2% PBL
No cluster designation	55K	IgA[c]	Serum IgA	<1% PBL
No cluster designation	23K	κ[d]	IgG, IgA, IgM, IgD, IgE	50–70 B cells
No cluster designation	23K	λ[d]	IgG, IgA, IgM, IgD, IgE	50–70 B cells
No cluster designation	28,34K	I2[a]	HLA-DR, B cells, monocytes, macrophages, activated T cells	7–15

[a]Coulter.
[b]Becton-Dickinson.
[c]Caltag.
[d]Tago.

analysis. The isolated mononuclear cells were fluorescently labeled by staining processes detailed below.

Antibodies

Commercial antibodies used for immunophenotyping analysis were used according to the manufacturer's instructions and are described in Table II. Monoclonal anti-Tn antibodies (BaGS-5 and BaGS-6) and anti-T antibodies (HT-8 and RS1-114) were prepared as previously described in ascites fluid and used diluted with buffered saline, with 0.2% sodium azide added [20–22]. Ascites were prepared by cultivating and expanding hybridoma cell lines prior to inoculation into mice. Host animals were kept in quarantine for 7 days, primed with pristane, and rested for 14 days. They were then inoculated with the appropriate hybridoma cells. Seven to 10 days later, harvesting of ascites fluid began. Harvested ascites fluid was processed to remove cell debris and then stored frozen at −70°C. Total protein determinations, concentrations of immunoglobulin, and results of electrophoretic analysis were documented. The anti-T antibody AH8-260 (catalog #86-5000) was obtained as a gift from David J. Rafter (Chembiomed, Edmonton, Alberta, Canada) and was made to red blood cells that had been desialylated (T-red blood cells). This monoclonal anti-T antibody was obtained as a 10% sucrose solution of 1 mg/ml and used diluted at 10 μg/10^6 cells. The mouse anti-Tn MAbs BaGS-5 and BaGS-6 were made to O MN Tn red blood cells, whereas the rat monoclonal anti-T MAb HT-8, was made to O MN red blood cells, desialylated [20,21]. The mouse anti-T Mab RS1-114 was raised against the human adenocarcinoma ascites of the lung cell line A549 [22].

Two secondary antibodies were used in this study. Fluorescein-conjugated $F(ab')_2$ fragment of a goat antimouse IgM (μ-chain-specific) was used for the mouse mAbs (Cappel, Organon Teknika Corporation, West Chester, PA catalog #1311-0201). Fluorescein-conjugated affinity-purified $F(ab')_2$ fragment of a goat anti-rat IgM (μ-chain-specific) was used for the rat mAbs (Cappel, catalog #1713-0201). These reagents were optimally titered for use to ensure maximum binding of each MAb to the cells.

An irrelevant ascites was used as the negative control (Cederlane Laboratories Limited, Hornby, Ontario, Canada via Accurate Chemical and Scientific Corporation, Westbury, NY). The control was obtained lyophilized from a BALB/c control ascites fluid with a protein concentration of 68.9 ± 2.4 mg/ml. This was reconstituted with 1.0 ml of distilled H_2O. This stock was diluted 1:10

and frozen in 1-ml aliquots at −70°C. The stock was used diluted 1:10 for control staining.

Immunostaining Techniques

All manipulations were performed on ice. For direct staining with conjugated mAbs for immunophenotyping, aliquots (100 μl) of a 1×10^7 cells per ml mononuclear cell suspension (1×10^6 cells) were added to each 12×75-mm plastic reaction tube. These cells were then mixed with the commercially prepared mouse MAbs shown in Table II for immunophenotyping. The cells were then incubated at 4°C for 30 min and washed twice with PBS containing 0.2% NaN_3 (PBS–NaN_3). The cells were then resuspended in 1 ml of PBS–NaN_3 and analyzed by flow cytometry.

For indirect staining, aliquots (100 μl) of the mononuclear cell suspension (1×10^6 cells) were added to each 12×75-mm plastic reaction tube (0.1 ml of 1×10^7 mononuclear cells per ml). Saturating antibody concentrations of diluted ascites anti-T and anti-Tn antibodies were added to each reaction tube, which were then incubated at 4°C for 30 min and then washed twice with PBS–NaN_3. Optimally diluted goat antimouse or antirat, $F(ab')_2$, fluorescein-conjugated polyclonal antibody specific for mouse or rat IgM heavy chains was added to individual reaction tubes. The tubes were incubated at 4°C for 30 min and washed twice with PBS–NaN_3. The cells were then resuspended in 1 ml of PBS–NaN_3 and analyzed by flow cytometry.

Negative controls tested on the isolated cells included the irrelevant ascites described above tested for anti-T and anti-Tn activity and found to be negative, as well as isotype-specific mouse immunoglobulin for the commercial reagents. In addition, all anti-T and anti-Tn MAbs were tested on lymphocytes from normal healthy adult volunteers. Positive controls to determine each antibody had activity consisted of K562 and Molt-4 cell lines [10].

Flow Cytometry

All analyses were performed using an EPICS V model 751 flow cytometer (Coulter Electronics, Hialeah, FL), equipped with a COHERENT(R) INNOVA 90-5 argon ion laser. Excitation of FITC was at 488 nm and emitted light was collected at 530 nm. All analyses were performed in a room with subdued lighting. The lymphocyte populations of interest were bit-mappped on a two-parameter forward angle versus 90-degree light scatter histogram. Cells were determined to be viable by propodium iodide exclusion [22]. Fluorescence data for each MAb were collected on 5,000 gated lymphocytes. The percentage of cells expressing surface antigens detected by the direct or indirect fluorescent labels was determined by setting cursor position channels with the use of the appropriate negative controls (lymphocytes stained with an irrelevant ascites) for the particular MAb. The staining was considered positive when more than 30% of the cells were stained. When 20–30% of the cells were stained, the staining was considered borderline positive.

Fluorescence Microscopy

All fluorescence microscopy and photography was performed with a Nikon Labophot biological microscope (Nikon Corporation, Melville, NY) equipped with an incident-light fluorescence illuminator Nikon FX-35 DX camera. Kodak Tri-X 400 ASA film was used and commercially developed.

Statistical Methods

Results are given as percent positive cells. The results are given as mean ± SEM. Student's *t*-test was applied to evaluate the data. One-tailed analyses were performed, and $P \leq 0.05$ was considered significant. All computations were performed using the StatWorks statistics program for the Macintosh from Cricket Software (Philadelphia, PA).

RESULTS

Clinical Profiles of Patients With B-CLL

Five untreated patients and one treated patient with B-CLL were studied. Patients were diagnosed on the basis of characteristic clinical, peripheral blood, and bone marrow findings. Single samples only were obtained from all patients except one (CLL #3) from whom three samples were obtained over time. Clinical characteristics of these subjects are given in Table I. At the time of the study, their ages were 55–78 years (mean age: 67.3 years). The duration of disease ranged from 2 months to 13 years, with a mean of 4.1 years. The patients had leukocyte counts of 12,500–55,000/mm^3 (mean: 28,450/mm^3), and the percentage of lymphocytes in peripheral blood was 44–99%. The absolute lymphocyte count ranged from 9,625 to 54,450/mm^3, with a mean of 25,100/mm^3. The hemoglobin values for these patients ranged between 10.1–16.3 g/dl, when a mean value of 14.2 g/dl. Their platelet counts ranged between 53,000–232,000/mm^3, with a mean value of 164,428/mm^3. Their symptoms were mainly fatigue, lymphadenopathy (LAP), and hepatosplenomegaly (HSM). Four of the patients had splenomegaly. The Rai stages of the patients ranged from 0 to 3 [24]. One patient (CLL #1) required therapy which included chlorambucil, prednisone, and cyclophosphamide. The same patient (CLL #1) died during the study.

Immunophenotypes of the Patients With B-CLL

Lymphocytes from the patients in this study were immunophenotyped (Table II) to determine the expression of markers commonly seen in this disease. The surface antigen profiles of the lymphocytes were consistent with

TABLE III. Immunophenotype of Lymphocytes From Patients With CLL

Patient sample date	Immunglobulin expression (% positive cells)					Immunophenotype (% positive cells)[a]										
	IgD	IgM	IgG	IgA	κ	λ	CD19 B4	CD20 B1	CD5 T1	T1B1	CD3 T3	HLA I2	CD10 J5	CD14 My4	CD11b Mo1	CD25 IL-2R	CD71 Transferrin R	CD45 KC56
CLL #1—3/11/91	9.4	10.2	2.7	3.1	8.6	67.0	90.5	37.0	78.7	54.4	18.9	97.6	16.3	28.5	28.2	7.5	8.2	70.7
CLL #2—5/2/91	70.1	49.1	3.2	4.8	25.6	59.3	60.3	95.3	98.8	96.1	5.0	94.2	17.2	27.8	24.2	11.0	17.0	95.6
CLL #3—4/9/91	18.2	43.0	12.5	4.5	1.0	63.8	70.7	84.3	13.1	6.3	13.7	82.3	52.0	59.8	6.2	8.5	0.7	80.0
CLL #3—5/8/91	14.3	18.1	16.1	5.3	12.0	65.6	88.0	95.5	18.2	14.8	27.1	93.9	60.5	89.3	61.9	46.5	36.8	93.0
CLL #3—7/30/91	21.8	19.9	11.1	4.4	9.9	30.5	91.8	94.5	16.9	13.2	16.0	92.4	60.0	88.4	59.7	67.4	23.7	88.4
CLL #4—5/2/91	65.8	58.9	8.1	4.9	93.7	4.4	85.4	90.6	96.9	88.4	10.4	88.7	25.2	30.7	23.2	7.8	10.7	94.3
CLL #5—4/18/91	47.0	51.9	1.1	5.1	82.1	1.9	69.9	76.9	34.4	5.2	30.8	71.4	41.1	72.5	47.2	28.7	15.4	91.3
CLL #6—7/30/91	69.3	55.3	3.8	4.7	5.5	88.0	81.2	82.3	96.6	79.8	15.6	83.9	21.1	42.4	46.0	6.7	5.6	92.6

[a]Determined by setting a dividing marker between positive and negative histograms of the same population.

B-CLL, as determined by immunofluorescence staining and flow cytometry, using a broad panel of MAbs reactive with leukocyte differentiation antigens (Table III).

Lymphocytes from patients had immunoglobulin on their cell surface, either IgM or IgD and IgM. Overall, B-CLL cells from four patients strongly expressed IgD, with 63.3 ± 5.4% of the cells positive for the molecule. Five samples were positive for IgM, with 51.6 ± 2.7% of the cells positive for the marker. One patient (CLL #3) initially showed expression of IgM on the surface of his cells; however, later samples were negative for the marker. IgG and IgA were not expressed (7.3 ± 1.9% and 4.6 ± 0.2%, respectively). Two of the patients expressed κ light chains, with 87.9 ± 5.8% cells positive, and four of the patients expressed λ light chains, with 62.4 ± 7.6% positive.

Lymphocytes from all patients had high levels of B4 (CD19), B1 (CD20), and I2 (HLA-DR), which is consistent with their B-cell malignancy. The pan-B-cell markers B4 (CD19) and B1 (CD20) were present on 79.7 ± 4.1% and 82.0 ± 6.9% of the cells, respectively. I2 (HLA-DR) was present on 88.1 ± 3.0% of the cells. Two patients failed to express T1 (CD5), which is characteristic of CLL. These patients' B cells were arrested at a slightly earlier stage of differentiation before the expression of CD5. The remaining patients expressing the marker T1 (CD5) expressed the marker on 92.8 ± 4.7% of their cells. One patient (CLL#2) showed high levels of this marker; however, this was due to the high percentage of T cells (34.4%) present in the sample, and not to the anomalous expression of the T-cell marker on the leukemic B cells. The coexpression of B1 (CD20) and T1 (CD5) was analyzed, and four of the six patients showed the anomalous expression of the T1 (CD5) marker on the B lymphocytes. The levels of T3 (CD3) are an indication of the quantity of residual T cells in the patient lymphocyte population. All patients showed low levels of residual T cells expressing the T3 (CD3) marker, with the exception of the above-mentioned patient with higher T-cell percentages.

The marker J5 or CALLA (CD10) was present in the two patients with arrest in earlier stages of maturation on 53.4 ± 4.5% of their cells. Four of the patients (67%) were positive for My4, a myeloid marker abnormally seen on B-CLL cells (63.8 ± 9.8% of their cells). Mo1 (CD11b) is a complement receptor; it was positive on the cells from three patients (50%). These cells expressed this marker on 53.7 ± 4.1% of the cells. The marker, interleukin-2 receptor (IL-2R) is an activation antigen and reflects the activation state of the cells. IL-2R was only positive on the cells from one patient (CLL #3), and 57.0 ± 10.5% of the cells were positive in two of three (67%) samples obtained from this patient. Only one sample was positive (13%) for transferrin receptor, another activation antigen. CD45 is a pan-leukocyte marker; it is detected by the antibody KC56. All the patient's lympho-

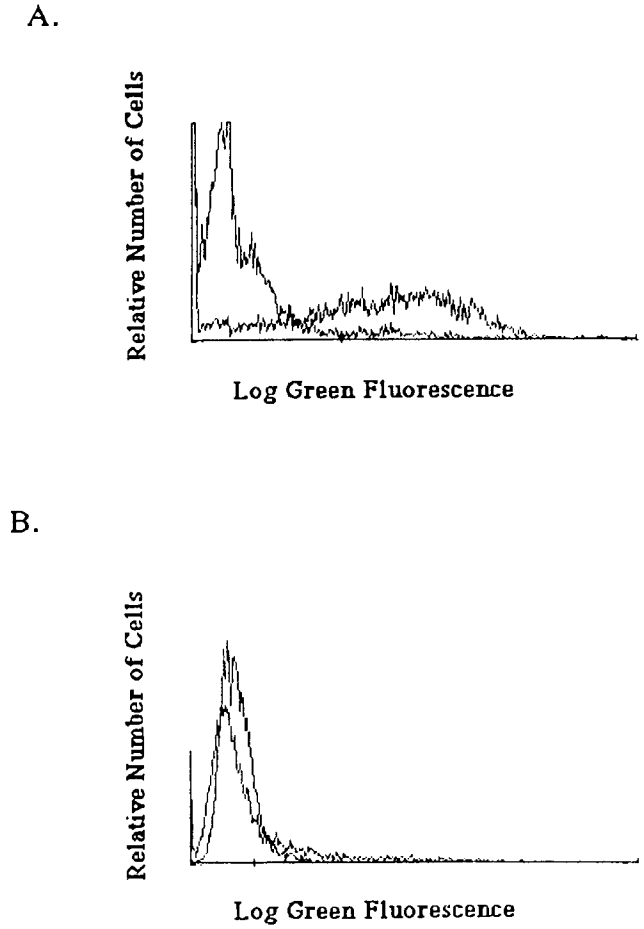

Fig. 1. Flow cytometric analysis of Tn expression. Single parameter frequency histogram comparisons from a patient with CLL (A) and normal controls (B) stained with anti-Tn antibody BaGS-5 relative to irrelevant ascites control. Each histogram represents integral green fluorescence on a 3-decade log scale with 256 channels, generated by measuring 5,000 viable cells.

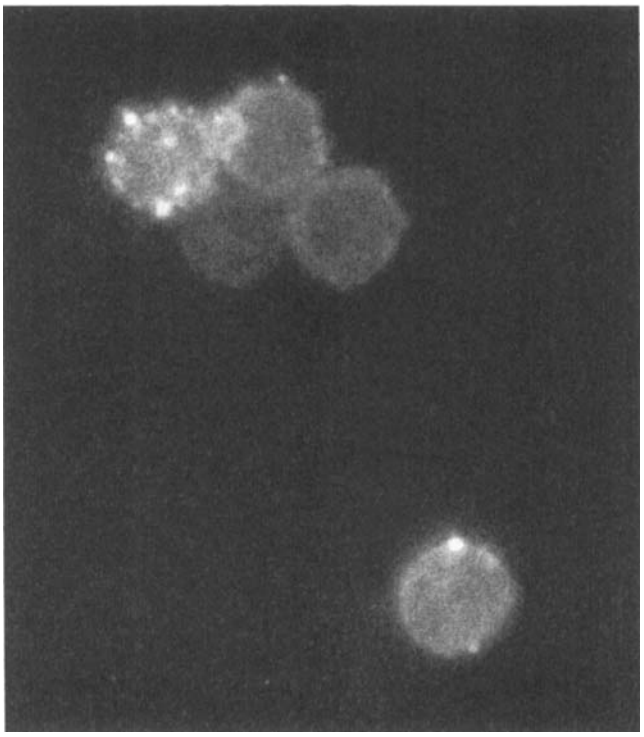

Fig. 2. Fluorescent microscopy analysis of chronic lymphocytic leukemia lymphocytes from patient CLL #2 showing positive staining for Tn antigen with BaGS-5 anti-Tn antibody. Note variation in staining intensity among cells consistent with flow cytometric analyses performed in this study.

cytes were positive, with 88.2 ± 3.0% of the cells positive for the marker.

T/Tn Epitope Analysis of Lymphocytes From Patients With B-CLL

The patients in this study were evaluated by flow cytometry for the presence of T and Tn antigens on their lymphocytes. A representative histogram of the B-CLL lymphocytes stained with anti-Tn antibody BaGS-5 is shown in Figure 1A. It can be seen that the amount of fluorescence of the cells relative to an irrelevant ascites control is quite different, the BaGS-5 antibody-stained cells being much brighter than the irrelevant ascites control. Fluorescence microscopy of the cells from patient CLL #2 stained with the antibody BaGS-5 confirmed a positive reaction (Fig. 2). Positive fluorescent staining was not apparent with the irrelevant ascites-stained cells, when viewed in a similar manner.

The results of the flow cytometric analysis can be found in Table IV. The percentage of lymphocytes from B-CLL patients that gave a positive staining reaction with BaGS-5 (anti-Tn) MAb ranged from 39.8 to 58.7%. Thus, an average 45.8 ± 2.1% of the cells from the leukemic B-CLL patient samples were positive. The anti-Tn antibody BaGS-6 gave very similar results, with an average of 44.0 ± 1.7% of the cells positive for eight samples from the six patients in this study. By contrast, lymphocytes from patient's cells appeared negative with the three antibodies used to detect T in this study. MAb RS1-114 showed only 10.9 ± 3.2% of the cells to be positive for the T EP. HT-8, which is also specific for the T EP only, showed 3.6 ± 0.7% of the cells as positive. The remaining anti-T MAb, AH8-260, was also essentially negative, with only 9.6 ± 4.2% of the cells positive for the T EP. One patient (CLL #6) showed some staining with both RS1-114 and AH8-260, which are both anti-T specific; however, with such borderline positive results, it is difficult to call these results positive for the T EP.

TABLE IV. T and Tn Expression on Lymphocytes From Patients With B-CLL

Patient sample date	Percent positive cells				
	BaGS-5 (Anti-Tn)	BaGS-6 (Anti-Tn)	RS1-114 (Anti-T)	HT-8 (Anti-T)	AH8-260 (Anti-T)
CLL #1—3/11/91	45.7	39.3	8.6	3.6	6.3
CLL #2—5/2/91	46.2	47.6	15.9	0.4	4.6
CLL #3—4/9/91	39.8	37.8	8.0	3.5	0.4
CLL #3—5/8/91	47.6	40.5	2.1	2.6	3.2
CLL #3—7/30/91	42.4	42.9	6.2	6.5	17.0
CLL #4—5/2/91	42.8	44.2	7.9	4.5	4.8
CLL #5—4/18/91	58.7	49.6	7.1	2.1	4.7
CLL #6—7/30/91	42.3	50.4	31.6	5.4	36.1

TABLE V. T and Tn Expression on Lymphocytes From Normal Healthy Volunteers

Volunteer	Percent positive cells				
	BaGS-5 (Anti-Tn)	BaGS-6 (Anti-Tn)	RS1-114 (Anti-T)	HT-8 (Anti-T)	AH8-260 (Anti-T)
Normal #1	13.4	18.4	2.0	1.8	N/Da
Normal #2	19.6	14.4	19.5	9.4	N/D
Normal #3	17.7	21.5	17.9	4.9	7.5
Normal #4	28.2	21.4	5.3	0.5	6.2
Normal #5	24.6	17.1	2.2	2.2	7.2
Normal #6	19.3	16.8	3.3	0.6	3.0

ND, not determined.

TABLE VI. Analysis of T and Tn Epitopes on Peripheral Blood Lymphocytes From Normal Individuals and Untreated Patients[a] With CLL

Antibody	Specificity	Percent positive cells[b]		
		Normal n = 6	CLL n = 8	*P*
BaGS-5	Anti-Tn	20.5 ± 2.1	45.8 ± 2.1	<0.001
BaGS-6	Anti-Tn	18.3 ± 1.1	44.0 ± 1.7	<0.001
RS1-114	Anti-T	8.4 ± 3.3	10.9 ± 3.2	NS[c]
HT-8	Anti-T	3.2 ± 1.4	3.6 ± 0.7	NS[c]
AH8-260	Anti-T	6.0 ± 1.0	9.6 ± 4.2	NS[c]

[a]These patients had either received no treatment or none during the previous 4 weeks prior to peripheral blood collection.
[b]Mean ± SEM.
[c]NS = not significant as by Student's *t*-test.

T/Tn Analysis of Lymphocytes From Normal Adult Volunteers

Lymphocytes from six normal healthy control were evaluated by flow cytometry for the presence of T and Tn antigen on their cells, using anti-T and anti-Tn MAbs. A representative histogram of the normal lymphocytes stained with anti-Tn antibody BaGS-5 is shown in Figure 1B. It can be seen that essentially no positive lymphocytes were observed, and the amount of fluorescence of these cells relative to an irrelevant ascites control is similar. Flow cytometric analysis of lymphocytes from normal controls with the MAbs to T or Tn showed essentially no expression (Table V). The staining pattern shows essentially a negative reaction with all the anti-T and anti-Tn antibodies used in this study. With the anti-Tn antibody BaGS-5, only 20.5 ± 2.1% of the cells were positive with this antibody. BaGS-6, another anti-Tn antibody showed that only 18.3 ± 1.1% of the cells were positive in the normal individuals. The anti-T antibodies showed a similar pattern of reactivity with the anti-T antibody RS1-114, giving 8.4 ± 3.3% of the cells positive and HT-8 showing 3.2 ± 1.4% of the cells as positive. The remaining anti-T, AH8-260 stained 6.0 ± 1.0% of the cells.

Comparison of Normal Volunteers With B-CLL Patients

The average expression of T and Tn EPs on the lymphocytes from the B-CLL patients versus the normal healthy controls was compared (Table VI and Fig. 3). The differences seen between the normal subjects and the B-CLL patients with the antibodies directed against the Tn EP, namely BaGS-5 and BaGS-6, were statistically significant ($P \leq 0.001$). The differences seen between the normal volunteers and the B-CLL patients were not significant, with the three antibodies used to detect the T EP (RS1-114, HT-8, and AH8-260).

DISCUSSION

The present study demonstrates that Tn EPs, but not T EPs, are expressed on the cells of patients with B-CLL. Previous studies using either immunohistochemistry or hemagglutination have hinted that T and Tn EPs may be

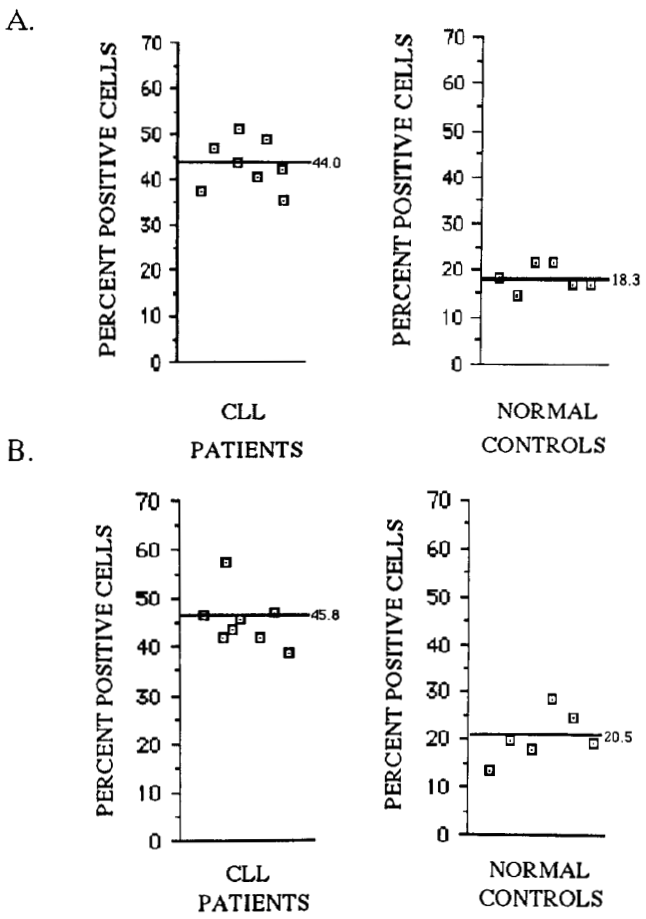

Fig. 3. Comparison of percentage of Tn-positive lymphocytes in controls versus patients with CLL with two anti-Tn antibodies: BaGS-5 (A) and BaGS-6 (B). Results are given as percentage of positive cells, as determined by flow cytometry. Numbers indicate mean values for the samples analyzed.

present on certain leukemia cells. Some of these studies have also used plant and mollusk lectins, which are not specific for a single carbohydrate-containing EP. Tn polyagglutinability has been reported in patients suffering from leukemia, indicating that their cells had the Tn EP exposed and thus the cells could react with the naturally occurring anti-Tn antibodies present in all human sera [4]. Previous reports indicated that these EPs should indeed be present. Our study further refined the analysis by adaptation to the flow cytometer, which allows large numbers of cells to be analyzed objectively, as well as the use of MAbs, which have a greater specificity than lectins for the EPs.

Both T and Tn are normally inaccessible to the immune system; however, all persons have anti-T and anti-Tn antibodies [1,2]. These antibodies are primarily due to the exposure to the intestinal flora [25,26]. These polyclonal antibodies, present in all human sera, have been used in studies of the presence of T and Tn EPs in CA [10,21]. Using monospecific polyclonal antisera, Springer et al. [11,27] showed in 1983 that murine DBA/2 ESb lymphoma cells have T and Tn specific structures on their surfaces. In 1985, using the polyclonal antisera, Springer et al. [10] showed the presence of T and Tn on various T-lymphoblastic and erythroleukemic cell lines; however, the B-lymphoblastic cell line RPMI-7666 was found to be negative. In another study done by Springer et al. [28], 3 out of 12 patients with leukemia or lymphoma showed a delayed-type hypersensitivity (DTH) response to T, indicating exposure to the EP. MAbs have also been produced against these EPs and used in the study of CA [18,26–30]. However, few studies have used MAbs to study the expression of T and Tn in leukemia [16].

Two lectins have been commonly used to detect T and Tn antigens—*Helix pomatia* (Roman snail lectin, HP), which reacts with Tn [31], and *Arachis hypogaea* (peanut lectin, PNA), which reacts with T [32], although neither is specific for those EPs alone. Thus, lectin binding is a hint of, but not proof for, the expression of these EPs. HP binding has been reported in CLL surface immunoglobulin-positive cells [33]. A small percentage of normal B lymphocytes also bound the HP lectin [34]. HP was found to bind to a major surface glycoprotein present on normal blood T cells and T leukemia cell lines [35]. Axelsson et al. [35] found that HP also bound CLL cells, a null cell leukemia cell line, an unidentified leukemia line, and a lymphoblastoid cell line of B origin and a stem cell lymphoma line; however, the glycoprotein was not found on various B cells at different steps of differentiation.

Several investigators have studied the binding of PNA to normal, reactive, and malignant lymphocytes. Rose et al. [36] found PNA to bind to human B lymphocytes in germinal centers and lymphomas. Reisner et al. [37] reported that PNA failed to bind to CLL cells but that it did react with Burkett's lymphoma cells. A study that analyzed PNA binding sites as differentiation markers of normal and malignant human lymphoid cells found that no CLL cells bound to the PNA [38]. In studies of ALL, those patients that had more than 15% of their cells bind the lectin were more likely to have relapses [39]. Recently Slesak et al. [40] reported slightly higher binding of PNA to B-CLL cells than normal B lymphocytes. Thus, there is little agreement as to the expression of T and Tn as determined by lectins binding to the B cells of the CLL patient.

Flow cytometry has also been used in previous studies of these markers. These studies include those that use both fluorescent lectins and MAbs. Coon and Weinstein [41] used lectins and flow cytometry to study CA. Orntoft and colleagues [42,43] also used lectins and flow cytometry in their studies of transitional cell CA and ploidy. Flow cytometry and HP lectin have been used to study

hematopoietic progenitors from patients with the Tn syndrome [44]. More recently, flow cytometry has been used in combination with MAbs to study T-antigen expression in human lung CA [22] and transitional cell cancer [45]. Finally, Bigbee et al. [46] used MAbs and flow cytometry to analyze erythrocyte populations in Tn syndrome blood, using MAbs to glycophorin A and the Tn antigen.

All patients analyzed in this study were diagnosed as B-CLL on the basis of characteristic clinical, peripheral blood, and bone marrow findings. All immunophenotyping data were consistent with the diagnosis of B-CLL [47,48]. None of the clinical prognostic factors or immunophenotypic markers correlated with the expression of these EPs (data not shown). As this leukemia may represent a single stage of differentiation, all the immunophenotypes were similar. Analysis for the T or Tn EPs showed a distinct pattern of expression. The Tn EP was expressed in the B-CLL patients as compared to normal, healthy adult volunteers. In addition, lymphocytes from normal controls were analyzed by two-color flow cytometric analysis using FITC-anti-Tn (BaGS-5 or BaGS-6) and either PE-anti-CD3 or PE-anti-CD20. Approximately one-third of the CD20 B-cell lymphocytes also had Tn antigens. By contrast, none of the CD3 T-cell lymphocytes had Tn antigen (data not shown). This finding suggests that Tn antigen is also expressed on some, but not all, peripheral blood lymphocytes. Since B-CLL is considered a malignancy of mature B lymphocytes, it is not unexpected that a subpopulation of these cells also has Tn antigen. However, the increased expression of Tn on B-CLL suggests that this antigen may be another marker for B-CLL and may lead to new insight on the growth and pathogenesis of these malignant cells. By contrast, the analysis for the T EP revealed no statistically significant expression in the B-CLL patients and the controls as determined by Student's *t*-test analysis.

We also studied nine other samples from five patients treated with chorambucil and prednisone, as well as with other standard treatment regimens for B-CLL during the previous 4 weeks (data not shown). We saw the same pattern of reactivity in both treated and untreated patients, and no statistically significant difference was found between these patients and those who were untreated, as determined by Student's *t*-test analysis. Thus, detection of the Tn EP does not appear to be affected by the treatment regimens used for the treatment of the B-CLL patients included in our investigation.

Previous studies done with B-CLL cells using lectins and immunohistochemistry as well as reports of Tn polyagglutinability in leukemia patients indicated the possible presence of the Tn EP on these cells. Using the MAbs and flow cytometry, we were better able to study the phenomenon. The previous lectin studies were hampered by the nonspecificity of the lectins for the desired EPs. Immunohistochemistry has the advantage of keeping the architecture of the tissues intact; however, the number of cells analyzed is limited. Using the MAbs and flow cytometry, we were able to be quite specific in detecting the EPs as well as in evaluating larger numbers of cells. Leukemias are ideally suited to the flow cytometer, as they exist as single-cell suspensions; and tissue disaggregation with enzymes is unnecessary. We performed flow cytometric analysis with the lectins HP and PNA (data not shown); however, the reactions were nonspecific with these lectins. MAbs were specific and had fewer background reactions. Some staining occurred with the MAbs in the normal controls. We attributed this to cross-reaction with similar EPs, such as the sialyl-Tn EP, and are currently investigating this phenomenon.

The present study shows that B-CLL lymphocytes express the Tn antigen as compared to normal B cells. The T EP was not found on these cells, however. As these EPs have been shown to be panCA markers that play an important role in the pathogenesis, diagnosis, and prognosis of CA, this finding may have the same importance in this leukemia as well.

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